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CONTRACT NO: DAMD17-89-C-9146

TITLE: A NOVEL SYSTEM FOR TESTING DERMAL AND EPIDERMAL TOXICITY IN VITRO

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REPORT DATE: February 15, 1990

TYPE OF REPORT: Phase I Final Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	15 February 1990	Phase I Final (8/15/89-2/15/90)

4. TITLE AND SUBTITLE
A NOVEL SYSTEM FOR TESTING DERMAL AND EPIDERMAL TOXICITY IN VITRO

5. FUNDING NUMBERS
Contract No.
DAMD17-89-C-9146

65502A
3P665502M802.BA.173
WUDA318720

6. AUTHOR(S)
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Marrow-Tech Incorporated
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT

The purpose of this Phase I project has been the modification and characterization of a dermal equivalent and full-thickness skin substrate as models for cytotoxicity and penetration studies. The dermal equivalent consists of mitotically and metabolically active fibroblasts and naturally secreted matrix proteins. A dermal/epidermal junction is present in the full-thickness skin model along with stratified keratinocytes and a stratum corneum. The work has involved (1) optimization of cell growth and uniform preparation of the three-dimensional (3D) substrates, (2) establishment of "standard operating procedures" to ensure quality control and reproducibility of test material, (3) adaptation of commonly utilized *in vitro* cytotoxicity assays for use with our substrates, (4) performance of standard tests using a series of known toxicant and irritants in conjunction with the 3D substrates, and (5) correlation of *in vitro* data with known *in vivo* toxicity and irritancy values.

14. SUBJECT TERMS	15. NUMBER OF PAGES
In Vitro Human Skin Model; Human Cells; Dermal Toxicity; Screening Tests; RA III; SBIR	16. PRICE CODE

17 SECURITY CLASSIFICATION OF REPORT	18 SECURITY CLASSIFICATION OF THIS PAGE	19 SECURITY CLASSIFICATION OF ABSTRACT	20 LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Project Summary

The purpose of this Phase I project has been the modification and characterization of a dermal equivalent and full-thickness skin substrate as models for cytotoxicity and penetration studies. The dermal equivalent consists of mitotically and metabolically active fibroblasts and naturally secreted matrix proteins. A dermal/epidermal junction is present in the full-thickness skin model along with stratified keratinocytes and a stratum corneum. The work has involved (1) optimization of cell growth and uniform preparation of the three-dimensional (3D) substrates, (2) establishment of "standard operating procedures" to ensure quality control and reproducibility of test material, (3) adaptation of commonly utilized in vitro cytotoxicity assays for use with our substrates, (4) performance of standard tests using a series of known toxicant and irritants in conjunction with the 3D substrates, and (5) correlation of in vitro data with known in vivo toxicity and irritancy values.

Research to date has concentrated on the utilization of the dermal and full-thickness skin equivalents in pilot testing with a modified neutral red (NR) assay, a mitochondrial dye uptake assay (MTT), a total protein test, a standard prostaglandon (PGE) release assay, and the lactate dehydrogenase (LDH) release assay. In addition, preliminary tests have been performed to illustrate the utility of the dermal substrate as a model for assessing the efficacy of growth factors in vitro. Important modifications made to the system include the development of an air-interface model which allows a simulation of the in vivo state and adaptation of the substrate to a standard 24-well kit form.

Results obtained during the Phase I contract period have indicated that the dermal and full-thickness skin models can be used effectively with a variety of assays. In all cases, results were reproducible. A direct relationship between cell damage or death and the concentration of toxicant utilized was seen upon exposure of the substrates to a panel of detergent-based compounds, alcohols, preservatives, and petrochemical derivatives. Values obtained with the in vitro test correlated well with known in vivo results. In addition, the dermal equivalent was used successfully in efficacy studies with various concentrations of fibroblast growth factors.

Work during this Phase I funding has indicated that both the dermal equivalent and full-thickness skin model can be used effectively to measure irritancy, cell damage, and cell death caused by various cosmetics, household products, and petrochemicals. In addition, the substrates may be utilized to measure specific cell response to various growth factors and pharmaceuticals. Applications of these products will

include (1) irritancy and toxicity screening of newly developed compounds, (2) penetration studies of water and lipid based products, and (3) efficacy testing of growth factors and pharmaceuticals.

SECTION I - PROJECT OBJECTIVES

Research on this Phase I contract involves the further modification and characterization of a three-dimensional (3D) human dermal model and a human full-thickness skin model which were developed in our laboratory. Work involves utilization of these substrates as dermal or skin equivalents in various in vitro studies. Protocols include use of a cultured dermal equivalent and a full-thickness skin equivalent as substrates in the following assays:

1. Expanded cytotoxicity assays utilizing a neutral red technique (NR), the 3-(4,5-dimethylthiazolyl) - 2, 5-diphenyl tetrazolium bromide (MTT) assay, and a modified total protein test.

2. Skin irritation assays using prostaglandin E_2 and Lactate Dehydrogenase (LDH) release as end points.

3. Efficacy studies for growth factors and pharmaceuticals.

SECTION II- WORK PERFORMED

During the six month funding period of this Phase I contract, we have accomplished the following objectives:

1. Established an inventory of neonatal and adult keratinocytes and fibroblasts. Modified the procedure for establishing and passing these cultures.

2. Modified both the dermal and full-thickness skin substrates so that they could be laser cut and used in 6, 12 or 24 well standard microtiter plates.

3. Modified the neutral red (N.R.) assay for use as a cytotoxicity test against the 3D substrates.

4. Developed a modified MTT assay for use as a comparison against the neutral red assay.

5. Modified the Total Protein test for use as an additional gauge for cytotoxicity.

6. Made a number of changes leading to optimization of a defined serum-free media for the skin substrate.

7. Utilized a standard radioimmunoassay for prostaglandin release to assess the ability of this assay to predict irritancy of a number of detergent-based compounds in conjunction with our 3D skin substrate.

8. Conducted pilot efficacy studies with fibroblast growth factors on our dermal equivalent to see if this substrate can be utilized in wound healing studies as well as in studies to measure induction of mitosis and extracellular matrix secretion.

9. Conducted pilot trials with toxicant treated full-thickness skin substrates to detect LDH leakage and release as an additional measure of cell irritancy.

10. Utilized the modified N.R. and MTT assays with a panel of known irritants to assess reproducibility of the substrates, the relationship between concentration of the chemicals and cell death and to compare in vitro cytotoxicity data with known in vivo Draize assay irritancy values.

11. Titration of a small panel of alcohols, preservatives, and ethanolamine through use of the N.R. and MTT assays; comparison of dermal and full-thickness skin in these assays.s

12. Modification of a membrane-based Millicell system to provide an air-interface and thus establish an easy system for studying penetration and permeability of both water and lipid-based compounds.

Results from each of the twelve areas above will be discussed separately.

SECTION III - RESULTS

1. Cell Inventory

During the six months of this contract, we have been successful at establishing primary culture from 20 neonatal foreskin samples (from routine circumcisions) and 6 adult tissue (from breast reduction surgery). Keratinocytes, melanocytes, and fibrablasts from each tissue sample were frozen at passage 1 and passage 3. We have, therefore, established an extensive inventory of epidermal and dermal cells which have been and will continue to be utilized to produce the 3D dermal and full-thickness skin equivalents.

The benefits of a cultured 3D skin substrate over cadaver skin include meeting the need for consistent and reproducible skin equivalents. Cadaver skin is obtained by a number of means at varying times after death, is cryopreserved by different methods, and is subjected to a number of stresses during transportation from the skin bank to the laboratory. These variables result in poor reproducibility between test samples. Establishing a 3D cultured skin substitute will provide a standardized, reproducible methodology for assessing irritancy, toxicity and rate of penetration of various test compounds. To optimize cell yield and quality control of substrate production, we have modified our preparation techniques for human keratinocytes and dermal fibroblasts and have established an extensive S.O.P. (standard operating procedures) manual. The following methods describe the modified versions of cell preparation protocols:

a. Tissue Preparation for Human Epidermal Keratinocyte and Dermal Fibroblast Cultures.

All reagents, solutions, instruments and plasticware must be sterile.

(1) Separation of Epidermis and Dermis.

1.1 Upon arrival, place the vial containing the skin in the refrigerator (2°-8°C) until ready for preparation.

1.2 In a sterile laminar flow hood, remove the skin from the collection vial with a sterile forcepts and place it in a 60 x 15 mm dish (Corning Cat. No. 25010 or equivalent) containing 6 ml of PBS w/o Ca++, Mg++ (Cellgro Cat. No. 21-031-LM or equivalent).

1.3 Remove the subcutaneous tissue with a sterile scissors and discard the subcutaneous tissue.

1.4 Transfer the skin to a sterile petri dish containing 70% ethanol solution. Cover and let stand for 1 to 2 minutes.

1.4.1 Foreskin: 60 x 15 mm dish (Corning Cat. No. 25010 or equivalent), 6 ml 70% ethanol

1.4.2 Adult Skin: 150 x 25 mm dish (Lab-Tek Cat. No. 4030 or equivalent), 40 ml 70% ethanol.

1.5 Aspirate the ethanol leaving the skin in the dish. Add PBS w/o Ca++, Mg++ to the dish.

1.5.1 Foreskin: 6 ml PBS w/o Ca++, Mg++.

1.5.2 Adult Skin: 40 ml PBS w/o Ca++, Mg++.

1.6 Cut the tissue lengthwise into strips or squares 2-3 mm wide with a sterile scalpel or scissors.

1.7 Aspirate the PBS w/o Ca++, Mg++ and add 0.02% EDTA to cover the tissue. Cover and let stand for 5 minutes.

1.8 Transfer the pieces of skin to a 60 x 16 mm dish(s) (Corning or equivalent) and add 6 ml of 0.25% trypsin. Immediately aspirate the trypsin solution.

1.9 Add 6 ml of 0.25% trypsin to the dish containing the pieces of skin.

1.10 Place the dish in a Hotpack incubator (or equivalent), 37°C, 5% CO_2, 90% + 5% relative humidity, for 2 hours.

1.11 Remove the dish from the incubator and peel the epidermis from the dermis with a sterile curved forceps. Transfer the dermal pieces to sterile 60 x 15 mm dish for fibroblast isolation (Step 3).

(2) Isolation of Keratinocytes.

2.1 With a sterile 5 ml pipet, remove the 0.25% trypsin solution, leaving the pieces of epidermis in the dish. Filter the solution through a short neck funnel (Nalgene Cat. No. 4252 or equivalent) lines with 2-ply sterile gauze (Johnson & Johnson Cat. No. 7623 or equivalent) into a sterile 50 ml conical centrifuge tube (Corning Cat. No. 25331 or equivalent) containing 5 ml of sterile fetal bovine serum (Hazleton Cat. No. 1210378 or equivalent).

2.2 Add 2 ml of 0.25% trypsin to the dish containing the pieces of epidermis.

2.3 Vigorously pipet the 0.25% trypsin solution and the epidermal sheets onto the bottom of the dish 5-10 times to break up the epidermis.

2.4 Filter the 0.25% trypsin solution containing the pieces of epidermis through the same gauze lined funnel and into the same 50 ml conical tube used in step 2.1.

2.5 Rinse the petri dish with 6 ml of PBS w/o Ca++, Mg++ and transfer to the same gauze lined funnel.

2.6 Centrifuge at 1000 rpm for 10 minutes in a IEC Centra 7 centrifuge (or equivalent).

2.7 Aspirate the medium from the centrifuge tube leaving the cell pellet.

2.8 Add 0.5 ml Keratinocyte Growth Medium (KGM) (Conetics Corp. Cat. No. CC3001 or equivalent) and gently tape the tube to resuspend the pellet.

2.9 Add 4.5 ml of KGM to the cell pellet and pipet the solution up and down 2-5 times to evenly mix the cells. Avoid formation of bubbles.

2.10 Transfer the 5 ml cell suspension to a sterile T25 flask (Corning Cat. No. 25100 or equivalent) and place the flask in a Hotpack incubator (or equivalent) equilibrated at 37°C, 5% carbon dioxide and 90% + 4% relative humidity.

2.11 Refeed the cultures with 5 ml KGM every 2-3 days.

2.12 When the cultures reach 70-90% confluence, trypsinize the cells for expansion or storage in liquid nitrogen.

(3) Dermal Fibroblast Explant Cultures.

3.1 Transfer the dermis to tissue culture treated petri disn(s). Place the pieces 1-2 cm apart with the dermal side down.

3.1.1 Foreskin: One 60 x 15 mm dish/foreskin.

3.1.2 Adult: 2-5 100 x 20 mm dishes/tissue.

3.2 Let the dish stand in the back of the sterile laminar flow hood with the lid ajar until the dermis is securely dried onto the dish (0.5 to 3 hours).

3.3 Gently add 6 ml of complete Dulbecco's Modified Eagle's Medium (DMEM). Place the dish(s) into a Hotpack incubator (or equivalent) at 37°C, 5% CO_2 and 90% + 4% relative humidity.

3.4 Refeed the cultures every 3-4 days with complete DMEM.

3.5 When the cells reach 70-90% confluence, trypsinize the cells for expansion or storage in liquid nitrogen.

b. Trypsinization and Expansion of Dermal Fibroblast Cultures.

1. Remove the dermal pieces from the primary cultures with a sterile forceps and aspirate the medium from the dish or flask.

2. Rinse the dish or flask with 2-10 ml of sterile PBS w/o Ca++, Mg++ (Cellgro Cat. No. 21-031-LM or equivalent).

3. Aspirate the PBS w/o Ca++, Mg++ and add an additional 2-10 ml of PBS w/o Ca++, Mg++. Let stand for 5 minutes and then aspirate the PBS.

4. Add 2-3 ml 1X trypsin/EDTA and tilt the dish or flask until the trypsin/EDTA solution covers the entire surface.

5. After 1 minutes, aspirate the trypsin/EDTA solution and using an inverted microscope (Nikon or equivalent), monitor the cultures for cell detachment. The dish or flask may require vigorous agitation to facilitate cell detachment.

6. Neutralize the trypsin/EDTA solution by adding 2-5 ml sterile fetal bovine serum (Hazleton Cat. No. 1210378 or equivalent) to the dish or flask.

7. Transfer the cell suspension to a sterile 50 ml conical centrifuge tube (Corning Cat. No. 25331 or equivalent) and centrifuge at 1000 rpm for 10 minutes in an IEC Centra 7 centrifuge (or equivalent).

8. Aspire the serum leaving the cell pellet. Add 1-5 ml of complete DMEM and gently tap the tube to resuspend the cell pellet.

9. At this point, either seed the cells into tissue culture vessels for expansion or prepare them for freezing.

   9.1 Seed all fibroblasts harvested from a primary in a 60x15 mm (Corning Cat. No. 25010 or equivalent) into a T25 flask (Corning Cat. No. 25100 or equivalent) without counnting the cells.

   9.2 Expand the fibroblasts harvested from a T25 by seeding all the cells into a T75 flask (Corning 25110 or equivalent) without counting the cells.

   9.3 Count the cells harvested from 70-90% confluent T75, T150 flasks or other culture vessels using a hemacytometer.

   9.4 Seed the cells into tissue culture flasks, dishes or equivalent at a density of 6-7 x 10^3 viable cells/cm^2 (i.e., 0.5 x 10^6 fibroblasts/T75 flask).

10. Maintain the cells in a Hotpack incubator (or equivalent) at 37°C, 5% CO_2 and 90% + 4% relative humidity.

11. Refeed the fibroblast cultures with complete DMEM medium every 3-4 days.

12. When the cultures reach 70-90% confluence, trypsinize the cells for expansion or prepare them for storage in liquid nitrogen.

(c) Trypsinization and Expansion of Epidermal Keratinocyte Cultures.

1. Aspirate the spent medium from the culture dish or flask.

2. Add 2-6 ml ATV solution, tilting the dish or flask to make sure the entire surface is covered.

3. Immediately aspirate the ATV solution.

4. Add an additional 2-6 ml of ATV to the culture dish or flask and let stand in the sterile hood for one minutes.

5. Using an inverted microscope (Nikon or equivalent), observe the cultures for cell detachment. Vigorous agitation of the culture vessel may be required to facilitate cell detachment.

6. Transfer the ATV solution containing cells to a 50 ml conical centrifuge tube (Corning Cat. No. 25331 or equivalent) containing 5 ml fetal bovine serum (Hazleton Cat. No. 1210378 or equivalent).

7. Add 2-6 ml sterile fetal bovine serum (Hazleton or equivalent) to the dish or flask to stop the trypsin in the ATV solution.

8. Vigorously pipet the serum onto the cell monolayer to harvest any cells remaining attached to the dish or flask.

9. Transfer the serum used to wash to culture dish or flask to the same conical centrifuge tube used in Step 6.

10. Centrifuge the cell suspension at 1000 rpm for 10 minutes in an IEC Centra 7 centrifuge (or equivalent).

11. Aspirate the serum from the cell pellet and resuspend the pellet 1-10 ml Keratinocyte Growth Medium (KGM) (Clonetics Corp. Cat. No. CC3001 or equivalent). At this point, either seed the keratinocytes for expansion or prepare them for storage in liquid nitrogen.

12. Seeding the keratinocytes.

    12.1 Seed keratinocytes harvested from a primary culture in a T25 flask (Corning Cat. No. 25100 or equivalent) into a single T75 flask (Corning Cat. No. 25110 or equivalent) without counting the cells.

    12.2 Count the keratinocytes harvested from T75, T150 or other culture vessels using a hemacytometer. Seed the cells at a density of 1-2 x 10^4 viable cells/cm^2 (i.e., 1 x 10^6 keratinocytes/T75 flask).

13. Maintain the cells in a Hotpack incubator (or equivalent) at 37°C, 5% CO_2 and 90% + 4% relative humidity.

14. Refeed the keratinocyte cultures with KGM (or equivalent) every 2-3 days.

15. When the keratinocyte cultures reach 70-80% confluence, trypsinize the cells for expansion or prepare them for storage in liquid nitrogen.

2. Modification of 3D Substrate for Use in Standard Microtiter Plates.

We have been successful at adapting our dermal and skin equivalents for use in standard 6, 12 and 24 well microtiter plates. Cells are grown on 8 x 15 cm nylon mesh pieces (as described in our original proposal). After four weeks of growth, a full dermis and multi-layered epidermis have formed and samples are cut with a medical grade laser (Texcel Corp. Mass.) on a teflon-coated steel template. The templates allow cutting of samples of any diameter and provide a system whereby (a) even cutting and minimum cell damage results, and (b) samples fall into microtiter wells positioned beneath the templates. This modification allows the rapid production of a reproducible substrate which can be utilized in standard assays and read on commonly available laboratory spectrophotometers. In addition, multiple wells of samples allow a number of control and test substances to be utilized in varying dilutions on essentially the same skin substrate.

3. Modification of the Neutral-Red (N.R.) Assay.

The neutral red (NR) assay is a commonly utilized method for assessing cytotoxicity of various products. NR is a weakly cationic dye that diffuses through plasma membranes and concentrates in lysosomes, binding electrostatically to anionic sites in these organelles. Thus, chemicals that damage lysosomal or plasma membranes will decrease the uptake or binding of the dye. It is commonly used on monolayer cultures in 96 well plates and, therefore, required modification for use on our dermal and skin equivalents. We have been successful at adapting this assay and have established the following protocol.

NEUTRAL RED ASSAY PROTOCOL FOR
DERMAL AND FULL-THICKNESS SKIN EQUIVALENTS

1. Place one pre-treated (i.e., HAc/FBS), pre-cut mesh each (no cells) into wells 1A, 1B and 1C of a 24-well plate. (1A will be used as an absolute blank; 1B and 1C will be used to determine background nonspecific binding [NSB] of neutral red to mesh). Add 2 ml DMEM complete ($DMEM_C$) per well to prevent desiccation of mesh.

2. Place one pre-cut mesh containing fibroblasts and keratinocytes into each test well of a plate containing 2 ml/well of DMEM complete.

3. Remove 2 ml of medium from each well and replace with 2 ml of test agent diluted in DMEM complete. Refeed untreated control mesh and mesh without cells with DMEM complete (wells 1A, 1B, 1C, 6A - 6D).

4. Incubate overnight at 37°C/5% CO_2, humidified.

5. Remove all spent media, then add 1 ml per well of a 1:80 dilution of 0.4% neutral red (NR) aqueous stock solution in DMEM complete. This yields a $NR/DMEM_C$ solution containing 50 µg/ml of dye. (Note: The $NR/DMEM_C$ solution is first centrifuged for 5 minutes at 3000 rpm to pellet undissolved dye crystals, then pre-warmed to 37°C before use). Add $DMEM_C$ without NR to well 1A.

6. Incubate for 3 hours at 37°C/5% CO_2.

7. Remove $NR/DMEM_C$ and wash each mesh 2x with 1 ml PBS + Ca, Mg, (about 2-5 minutes each).

8. Wash each mesh 1x with 1 ml Wash/Fix solution (0.5% formaldehyde, 1% calcium chloride) for one minute only at room temperature. Do this 4-8 wells at a time, since prolonged exposure to formaldehyde will extract the dye prematurely.

9. Remove Wash/Fix solution, then add 2 ml Solvent (1% acetic acid in 50% aqueous ethanol) at room temperature for 60 minutes. This will extract the dye from lysosomes of cells within the mesh into the supernatant. (Note: the amount

extraction is proportional to the number of viable cells within the culture).

10. Transfer 200 ul aliquots of the reddened spent solvent from each well to a 96-well plate and read the plate on a microplate reader at 540 mm (filter 4 on Dynatech) blanking to well 1A which was not exposed to neutral red. (Wells 1B and 1C yield NSB data of NR to mesh; this value can subsequently be manually subtracted prior to the calculations described below).

11. After subtracting the mean OD_{540} of wells 1B and 1C from the OD_{540} reading of each dilution of test agent and of the untreated control mesh, "% of untreated control" can be calculated as follows:

$$\frac{OD_{540} \text{ of test agent dilution}}{OD_{540} \text{ mean of untreated control mesh}} \times 100 = \% \text{ of untreated control}$$

12. Plot the "% of untreated control" for each dilution of a given test agent on the y-axis vs. the concentration of test agent (in µg/ml) on the x-axis and determine the NR-50 endpoint (i.e., that concentration of test agent which reduced NR dye incorporation by 50% compared with untreated control cultures).

13. The NR-50's for test agents within a given chemical class may then be used to rank-order their relative toxicities.

4. Modification of the MTT Assay.

The MTT assay is a widely utilized assay for measuring cytotoxicity based on mitochondrial uptake of 3-(4, 5-dimethyl thiazol) - 2,5-diphenyl tetrazolium bromide and processing of this chemical during an active oxidation-reduction reaction. It is a measure of cellular viability and activity and can be used effectively in parallel with the N.R. assay in which we see lysosomal staining with the red dye. Comparable data with both of these assays will help to establish a more definite range of *in vitro* data for a large panel of irritants and toxicants. During the phase I contract period, the following MTT assay protocol has been developed and successfully utilized.

ADAPTED MTT ASSAY PROTOCOL FOR 3D SUBSTRATES

1. Place one pre-treated (i.e., HAc/FBS), pre-cut mesh each (no cells) into wells 1A,d 1B and 1C of a 24-well plate. (1A will be used as an absolute blank; 1B and 1C will be used to determine background nonspecific binding [NSB] of MTT to mesh). Add 2 ml DMEM complete DMEM complete ($DMEM_C$) per well to prevent desiccation of mesh.

2. Place one pre-cut mesh containing fibroblasts and keratinocytes into each test well of a plate containing 2 ml/well of DMEM complete.

3. Remove 2 ml of medium from each well and replace with 2 ml of test agent diluted in DMEM complete. Refeed untreated control mesh and mesh without cells with DMEM complete (wells 1A, 1B, 1C, 6A - 6D).

4. Incubate overnight at 37°C/5% CO_2, humidified.

5. Remove all spent media, then add 1 ml per well of DMEM complete containing 0.5 mg/ml MTT. (Note: The MTT/$DMEM_C$ is first centrifuged for 5 minutes at 3000 rpm to pellet undissolved dye crystals, then pre-warmed to 37°C before use). Add $DMEM_C$ without MTT to well 1A.

6. Incubate for 4 hours at 37°C/5% CO_2.

7. Remove MTT/$DMEM_C$ and wash each mesh 2x with 1 ml PBS + Ca, Mg, (about 2-5 minutes each).

8. Remove the second PBS wash, then add 2 ml isopropanol or DMSO at room temperature for 60 minutes. This will extract the formazan from mitochondria of cells within the mesh into the supernatant. (Note: the amount of MTT taken up by a cell culture and subsequently released by solvent extraction is proportional to the number of viable cells within the culture).

9. Transfer 200 ul aliquots of the blue spent solvent from each well to a 96-well plate and read the plate on a microplate reader at 540 nm (filter 4 on Dynatech) blanking to well 1A which was not exposed to MTT. (Wells 1B and 1C yield NSB data of MTT to mesh; this value can subsequently be manually subtracted prior to the calculations described below).

10. After subtracting the mean OD_{540} of wells 1B and 1C from the OD_{540} reading of each dilution of test agent and of the untreated control mesh, "% of untreated control" can be calculated as follows:

$$\frac{OD_{540} \text{ of test agent dilution}}{OD_{540} \text{ mean of untreated control mesh}} \times 100 = \% \text{ of untreated control}$$

11. Plot the "% of untreated control" for each dilution of a given test agent on the y-axis vs. the concentration of test agent (in µg/ml) on the x-axis and determine the MTT-50 endpoint (i.e., that concentration of test agent which reduced MTT turnover by 50% compared with untreated control cultures).

12. The MTT-50's for test agents within a given chemical class may then be used to rank-order their relative toxicities.

5. Modification of the Total Protein Test.

This assay procedure measures the growth inhibitory potency of test agents by determining their ability to reduce the accumulation of total cell protein. Cells cultured at low density in 96-well plates are treated with test agents for 24 hours, washed with a buffer, lysed with NaOH, and then treated in situ with a protein-binding dye. The plates are scanned in a microplate reader, which automatically measures the optical density of the dye bound to cell protein in each well, and then transfers the data to a

microcomputer programmed to convert the results to dose-response curves. The concentration of test agent causing a 50% reduction in protein accumulation relative to mock-treated controls (P50) is used as a comparative endpoint. The mechanistic basis of this assay is very general; growth inhibition is an early indicator of cell damage caused by virtually all toxic processes. We worked to modify this test prccedure so that it can be used effectively with our 3D substrate. Repeated assays with various concentrations of sodium dodecyl sulfate (SDS) were performed to set standard dose curves. The following procedure resulted frcm this pilot study:

MODIFIED TOTAL PROTEIN TEST

1. Incubate the mesh cultures overnight with test agents diluted in TC medium.

2. Remove test agents, wash mesh 4 x with PBS.

3. Remove final PBS wash, then add 2.5 ml 0.1N NaOH to each mesh for 60 minutes at room temperature agitating on shaker platform.

4. Remove mesh from the wells, then transfer 50 ul from each of the wells into quadruplicate wells of a 96-well plate. Also put 50 ul of 0.1N NaOH into quadruplicate wells for use as a blank.

5. Add 50 ul of various BSA dilutions (made in 0.1N NaOH) to quadruplicate wells (0, 10, 20, 40, 60, 80, 100, 120, 140, 160, 200 and 400 µg/ml) to generate the standard curve.

6. To each well containing 50 ul, add 200 ul of a 1:3 aqueous dilution of the BioRad Protein Reagent Concentrate (Bradford Reagent) for 5 minutes at room temperature.

7. Read the plate at 650 nm (reference wavelength 405 nm).

8. Using a quadratic curve fitting mode, determine the amount of protein in each of the test samples.

9. Calculate percent of untreated control for each of the dilutions of test agent and plot percent of untreated control on the y-axis and concentration of test agent on the x-axis.

The endpoint is yet to be decided upon, since we may not consistently reach 50% reduction in protein content due to the deposition of collagen and other extracellular matrix proteinaceous material within the mesh. A better endpoint here might be P-75 (25% reduction in protein content). Additional experiments will be performed in conjunction with the NR and MTT assays to better determine endpoints and significance of variability between the titration curves from the various assays.

6. Optimization of Serum-Free Media for Keratinocyte Growth.

Standard serum-free keratinocyte media can be purchased (Clonetics, San Diego, Calif.), but is expensive and often shows variability between different lot numbers. Since we are concerned with developing the most standardized total substrate growth as is possible, we have made modifications to this media, tested it for reproducibility and efficiency, and have incorporated production of this media into our S.O.P. The modified keratinocyte media is prepared as follows:

To one liter MCDB 153 (purchased from Hazleton Labs) add Amino Acid Supplement.

His	0.372 grams	
Ile	0.09	
Met	0.134	in 100 ml MCDB 153
Phe	0.148	
Trp	0.092	
Tyr	0.013	

Fatty acid supplement:

Need 10 µg/ml in medium or 10 mg/liter
Stock solution is 50 mg each in EtOH. Palmitic acid
5 ml Linoleic acid
Arachadonic acid
Stock solution is 10 mg/ml = 10^3X. Add 1 ml/L medium.

Hydrocortisone supplement:
Hydrocortisone was added to the fatty acid solution.
M.W. = 362.47 .000181 g/L = 0.5 µM

Ethanolamine MW = 61.08
Want 0.2 µM = 12.2 ml/L or 12.2 ml/L

Phosphoethanolamine
MW = 141
Want 0.1 µM = 14.1 mg/L
Stock solution = 141 mg/10L
141 mg in 5 ml. Add 0.5 ml/L

Mouse epidermal growth factor reconstituted 100 mg/ml in PBS with 1 mg/ml BSA.
10 ng/ml in medium
10 µg/L = 0.1 ml of 100 mg/ml

Insulin reconstituted in 1.0 ml sterile H_2O 100 µg/m.

5 µg/ml in medium
5 µl/L = 50 ml

Bovine Pituitary extract (Clonetics) 2 ml/500 ml medium.

7. Prostaglandin Release Assay.

Release of PGE_2 has been used as an indicator of cell membrane damage and irritancy. This assay has gained support as a reproducible means of predicting irritancy of a variety of lye-based compounds and is currently utilized as an assessment of cell damage with new product development in the household product industry. Over the next six months, we will continue to develop a prostaglandin release assay based on enzyme staining techniques (modified Enzyme Linked Immunosorbent Assay (ELISA method). Experimentation over the last month has involved use of the PGE_2 assay kit from Boehringer Mannheim. This pilot work has allowed us to assess the utility of the 3D substrate in such a radioimmune assay (RIA). Briefly, the protocol is as follows:

PGE_2 RELEASE ASSAY

1. Add test agent diluted to various concentrations in appropriate tissue culture medium directly to wells containing fibroblast and keratinocyte mesh cultures. Add tissue culture medium without toxicants to control meshes to establish baseline PGE_2 release.

2. Incubate for 30 minutes to 1 hour.

3. Examine "spent" cell culture supernatants for release of PGE_2 from mesh cultures by specific radioimmunoassay or enzyme immunoassay.

4. Quantify released PGE_2 versus a standard curve using various dilutions of purified PGE_2 in the Enzyme Immuno Assay (EIA) or RIA.

For pilot studies, three concentrations of SDS were utilized: 0.0001%, 0.001%, and 0.01%. The same concentrations were utilized in our neutral red assay, MTT assay, and the LDH assay to allow comparison of results. As can be seen on the following bar graph (Figure 1), the amount of PGE2 (PCG/0.1 ml) released increased with the increasing concentration of SDS. This data is complementary to the neutral red data on the 3D substrate with a viability decrease seen with increasing concentration in the cytotoxicity assay. The lowest concentration of SDS gave readings which were lower than the untreated controls. This finding can be explained by the fact that low concentrations of SDS cause a temporary strengthening of

the cell membrane structure by binding to phospholipid membrane components.

8. Growth Factor Effficacy Studies.

The ability for various growth factors to initiate cell division was studied using thymidine incorporation into adherent cell populations. Substrates utilized included neonatal fibroblast dermal equivalents (from foreskin) and adult dermal equivalents (from breast reduction surgery) supplied by Marrow-Tech. All cells utilized were applied to the mesh after 3-4 passages in monolayer.

Methods:

1. Add growth factor diluted to ten increasing concentrations in tissue culture medium directly to wells containing dermal mesh cultures (24 well plates). Control wells receive medium alone to establish baseline thymidine incorporation. Substrates are incubated for 24 and 48 hour intervals. Assays are run in triplicate at 37°C and 5% CO_2 in a humidified chamber.

2. Eighteen (18) hours prior to harvesting 10 uCi of tritiated thymidine is added to each of the wells. Wells are rinsed with 50 ul of PBS.

3. Cells are harvested and scintillation fluid added. All data is recorded in triplicate with thymidine incorporation being proportional to the rate of cell proliferation.

Dermal equivalents were exposed to beta Fibroblast Growth Factor (bFGF) for 24 and 48 hour time periods. bFGF concentrations included: 2, 4, 6, 8, and 10 ul/ml. Neonatal and adult dermis were analyzed. As can be seen in the two following graphs, there was a linear response in cell turnover number to the concentration of FGF in both neonatal and adult substrates after 24 hour exposure (Figure 2). After a 48 hour incubation time, a far greater response was noted in neonatal dermis (Figure 3). This finding can be explained by the ability of neonatal cells to divide more frequently than adult cells and by a greater concentration of receptors for growth factors being present on neonatal cells.

A system to assay cell turnover and matrix protein secretion in vitro would be an extremely valuable tool in wound healing

studies. In addition, such a study would allow researchers a means of (a) assessing efficacy of new growth factors and healing-promoting agent, (b) measuring toxicity and prevention of healing by implantable devices and steroid-based compounds, (c) measuring growth-factor production by genetically engineered cells, and (d) establishing wound healing models from diseased patients (such as diabetics) where healing is often impaired.

9. LDH Release Assay.

The presence of lactate dehydrogenase is an indication of cell membrane damage. Leakage of this enzyme occurs upon exposure of the cells to various toxicants and irritants and indicates cell damage before cell death. Standard concentrations of SDS were tested against our full-thickness skin substrate utilizing the following methodology:

LDH RELEASE ASSAY

1. Add test agent diluted to various concentrations in tissue culture medium directly to wells containing fibroblast and keratinocyte mesh cultures. Add tissue culture medium without toxicants to control meshes to establish baseline LDH leakage.

2. Incubate at 12 or 24 hour intervals.

3. Examine "spent" cell culture supernatants for the presence of lactate dehydrogenase enzyme by adding a Tris-buffered substrate containing lactic acid, chloride (INT), phenazine methosulfate (PMS), and nicotine adenine dinucleotide (NAD). The observed color change can be observed at 490 nm and can be compared versus the standard curve of known concentration of LDH.

$$\text{Lactate} + \text{NAD} \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH}$$

Results of this pilot study are seen on the following bar graph (Figure 4). A release of LDH seen was proportional to the concentration of SDS utilized. Difficulty in reproducibility was initially noted among various substrates after treatment with 0.001% of SDS only (see standard error bar) after these individual assays were conducted.

The assay was repeated on a panel of full-thickness skin substrates established from three additional tissue sources. Results from these assays are presented below:

LDH RELEASE (U/L)*

	Substrate 1	Substrate 2	Substrate 3
control	18 ± 2	21 ± 3	22 ± 2
0.0001% SDS	31 ± 3	30 ±2	33 ± 4
0.001% SDS	79 ±7	76 ± 5	81 ± 8
0.01% SDS	124 ± 4	122 ± 8	131 ± 10

* Each point run in triplicate.

It is evident from this expanded pilot trial that the LDH release assay can be utilized on our 3D substrates. Reproducibility is good (low standard error) and a direct correlation is evident between concentration of test agent and LDH.

The variability noted during the first pilot study has been attributed to inconsistency of substrates between triplicate wells. Production of an S.O.P. and extensive quality control testing of substrates prior to assay has significantly reduced this variability. We will continue to utilize the PGE assay with an expanded panel of known irritants to further assess the ability of our substrate to be used as a physiological prediction of irritation in vivo.

10. Use of the NR and MTT Assays.

During this Phase I contract study, we have run a broad panel of irritants and toxicants on our full-thickness skin equivalent utilizing the modified NR and MTT assays outlined earlier in this report. In general, we have been able to make the following observations:

(a) The NR and MTT assays can be utilized to give reproducible dose-response titration curves with the 3D skin substrates.

(b) Various irritants and toxicants can be utilized effectively.

(c) NR and MTT are indicative of in vivo results obtained from standard Draize tests.

Results showing the staining of cells which result from use of these assays have been included in monthly reports during this contract period. Dye uptake (NR test) and mitochondrial staining (MTT assay) are proportional to cell viability and could be visually

correlated to the concentration of toxicant utilized. Automated spectrophotometric analysts of the elected fractions provides an excellent quantification of cell toxicity. The following graphs represent the values obtained with the NR and MTT assays when various irritants were applied to our full-thickness skin model. Test agents include a coded panel of known irritants provided from the Soap and Detergent Association (SDA), commercial shampoos and household agents.

These results were reproducible and could be correlated, in general, with in vivo Draize scores, as seen below.

IN VIVO VERSUS IN VITRO COMPARISONS

SDA COMPOUND CODE	DR. SCORE	NR-50 EXPT #1	NR-50 EXPT #2	COMPOSITE NR50
212	6.0	3878	NT	3878
217	4.0	593	787	650
222 +	67.1	4191	NT	4191
237 °	33.3	>5000	NT	>5000
242	26.0	218	199	208
244	10.1	>5000	8073	8073
264	27.7	125	156	142
268	42.5	202	198	200
273	5.3	3746	6358	5571
216	65.8	20	22	21
246	4.0	580	591	586
255	60.0	24	26	25
272	21.0	128	164	146
291	82.0	15	12	13.5

NT = not tested
\+ = alkaline
° = acidic

Figure 18 shows a plotting of the log of the average NR-50 values against average maximum Draize scores. It can be noted that a good correlation exists for most irritants; compounds resulting in an immediate pH change, such as acetic acid or ammonia, cannot be utilized reproducibly.

Both the NR and MTT assays have been utilized with our system to give good titation curves with the compounds tested. The MTT assay has proved to be more sensitive, primarily due to the lack of background staining onto the nylon mesh material (see appropriate

monthly report). We plan to continue utilizing a larger panel of irritants on our system, utilizing both the NR and MTT assays in order to get a more significant evaluation of the in vitro data obtained.

11. NR and MTT Assays: Testing of Alcohols and Preservatives.

During the Phase I contract period, pilot studies have been performed to test the suitability of our substrates as models for toxicity and irritancy prediction in a variety of compounds. In addition to the range of detergent-based compounds tested, we have tested the following groups of agents: alcohols, preservatives, petrochemicals and additives. Sample results from such tests can be seen in Figures 19-27.

(a) Alcohols. With both the NR and MTT assays, we were able to get reproducible results when testing methanol, ethanol and propanol on our full-thickness substrates. In both cases, a different range of toxicity was noted, with propanol testing most toxic with the MTT assay and ethanol proving most toxic with the NR assay.s In order to see if our 3D substrate was resulting in the discrepancy between assays we utilized, the NR and MTT assays and this panel of alcohols on monolayer cultures of keratinocyte. The following results were obtained:

Neutral Red

ethanol.....26,854 µg/ml (N=12 assays)
methanol.....34,500 µg/ml (N= 7 assays)
propanol.....23,400 µg/ml (N= 5 assays)

This would give the following toxicity ranking:

propanol>ethanol>methanol

MTT

ethanol.....27,000 µg/ml (N=12 assays)
methanol.....24,800 µg/ml (N= 7 assays)
propanol.....24,300 µg/ml (N= 5 assays)

This would give the following toxicity ranking:

propanol>methanol>ethanol

The LD-50 values for each of these alcohols are:

ethanol.....10.6 g/kg
methanol.....not listed
propanol.....5.8 g/kg

This would rank propanol as more toxic than ethanol.

These results would indicate that the MTT assay would be most sensitive in predicting toxicity values of alcohol-based compounds, with monolayers as well as with our 3D substrates. Work will continue on a broader panel of alcohols at multiple dilutions with NR, MTT and total protein compared as assay protocols.

(b) Ethanolamine and Hydrocarbon. Ethanolamine is a viscous, hygroscopic liquid with an amoniacal odor. It is used:

(1) by the petrochemical industry to remove CO_2 and H_2S from natural gas and other gases,
(2) in polishes, hair waving solutions and emulsifiers,
(3) as a softening agent for hides,
(4) as a dispersing agent for agricultural chemicals,
(5) by reaction with other substances to form an accelerator in the manufacture of antibiotics. Also, as an oleate, it is used therapeutically as a sclerosing agent.

The LD-50 of ethanolamine in rats is 10.20 g/kg.

Figure 22 indicates the NR titration curve obtained with ethanolamine on our full-thickness skin equivalent. A dose-related curve is seen with limited concentration points used. The 3D substrates will be further used to test a broad panel of petrochemical and agricultured chemical additions.

Chlordane and other chlorinated derivatives are common solvents, degreasers and lubricants and are used in the manufacture of many herbicides. Various triorgenotins are employed as biocytes. Chlordane (Figure 23) and tributyltinchloride (Figure 24) were run on both our dermal and full-thickness skin equivalents to test the utility of our substrates in predicting toxicity of these common

environmental poluters. It is evident from the titration curves that a dose-related response is seen with both classes of compounds on both types of test substrates. Repeated testing showed that results were reproducible.

Herbicides, pesticides and petrochemical additives to be tested in the future include:

malathion
carbaryl
aldicarb
zinc dithiophosphate (lube oil additive)

A small panel of preservatives were tested in a pilot run against our full-thickness skin with the NR assay. Titration curves for benzallonium chloride (Figure 25), thimerosal (Figure 26) and formaldehyde (Figure 27). The dose-response seen was consistent between triplicate wells that were tested. An expanded range of concentration should be utilized with both the NR and MTT assays to better correlate the dose response and offer baseline data for comparison. Additional preservatives which should be tested include sodium azide and benzoic acid.

12. Adaptation of MilliCell Chambers and 3D Dermal Equivalent for Penetration Tests.

In order to provide a reproducible means for measuring the penetration of water and lipid-based compound in vitro we have adapted a 6-well MilliCell membrane system for use with keratinocytes and our dermal equivalent. Human keratinocytes are seeded onto the MilliCell membrane and dermal equivalent (laser-cut) is sandwiched against the opposite membrane surface. This allows a full-thickness skin equivalent composed of (a) stratified keratinocytes on a membrane, and (b) dermal fibroblasts and collagen on a mesh. The two-layered equivalents in the MilliCell are utilized with an adapted diffusion chamber consisting of donor and receiver reservoirs and a water jacket. MilliCell diffusion chambers are prepared by attaching the air lines and heating with a water jacket to 37°C. (The air bubbled through the solutions provides mixing in the donor and receiver reservoirs. This minimizes the aqueous boundary layer formed at the surface of the MilliCell.)

Cultured skin equivalents are washed with Hank's balanced salts solution (HBSS) and then transferred to the diffusion chamber. The

donor and receiver reservoirs are each filled with 3 ml HBSS. Penetrant is then added to the donor side of the chamber. The chamber is maintained at 37°C with a water jacket. At 15-30 minute intervals, 0.1 ml samples are removed from the receiver side of chamber and an equal volume HBSS is added to the chamber to replace the sample volume. Penetrant in the receiver chamber is then quantitated by appropriate means (radioactivity, UV absorbance, etc.).

Data is expressed as a permeability coefficient (P) in cm/sec calculated according to Fick's law:

$$P = \frac{Vr \; dC/dT}{A \; \Delta C}$$

Vr = Volume of the receiver chamber fluid

ΔC = Concentration gradient of the penetrant

dC/dT = Flux of penetrant

A = Diffusional area

This culture system device is water-tight and simulates the penetration boundary of cornified human skin. This modified system will allow the penetration studies of many classes of compounds including cosmetics, herbicides, pesticides and petrochemicals.

SECTION IV - TECHNICAL FEASIBILITY AND APPLICATIONS.

Work during this Phase I contract has resulted in the standardization of the production of the dermal and full-thickness skin equivalents and adaptation of these substrates too commonly used to assay. Tests adapted for use in this study include measurements for:

1. Cytotoxicity (NR, MTT, total protein),
2. Cell damage (LDH; PGE_2)
3. Growth changes and growth factor activity (bFGF measurements).

A broad range of compounds were tested, including:

1. Detergents
2. Common household products and shampoos
3. Alcohols

4. Petrochemicals
5. Preservatives

The substrate was also adapted for use in permeability and penetration studies.

Results obtained during this contract have indicated that the dermal equivalent and full-thickness skin equivalent developed in our laboratory can successfully be adapted for use in screening a variety of compounds for toxicity and efficacy endpoints. The substrates can be utilized for testing household products, cosmetics, petrochemicals, herbicides, pesticides and pharmaceuticals.

GN:bb:5.01
8/15/89-2/15/90

EVALUATION OF MARROW -TECH FIBROBLAST/KERATINOCYTE CULTURES AFTER SDS TREATMENT

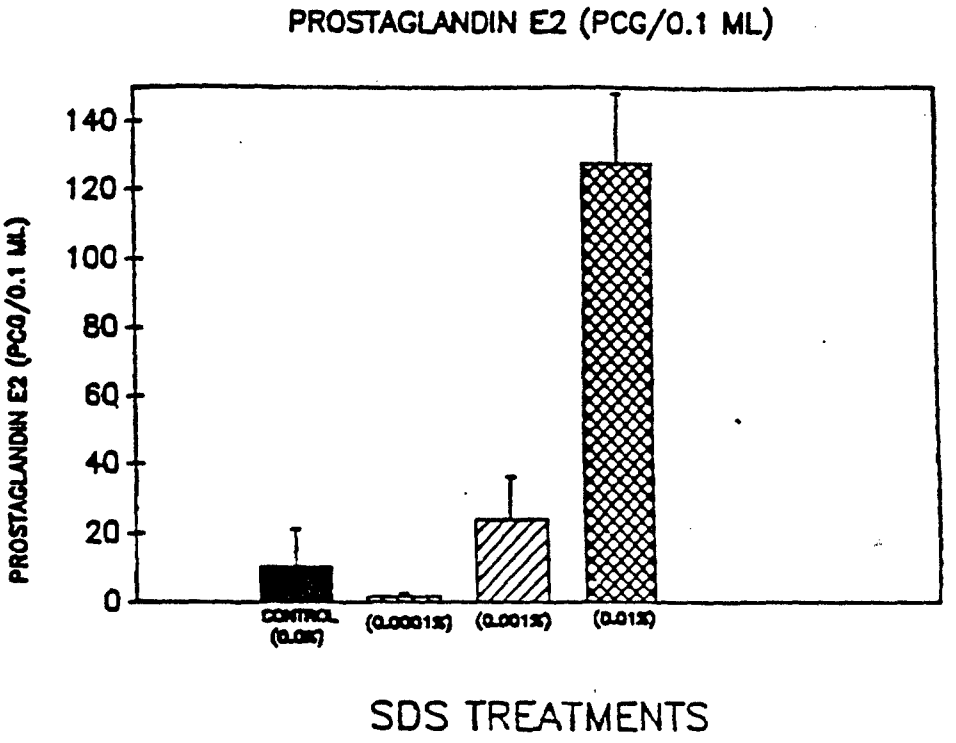
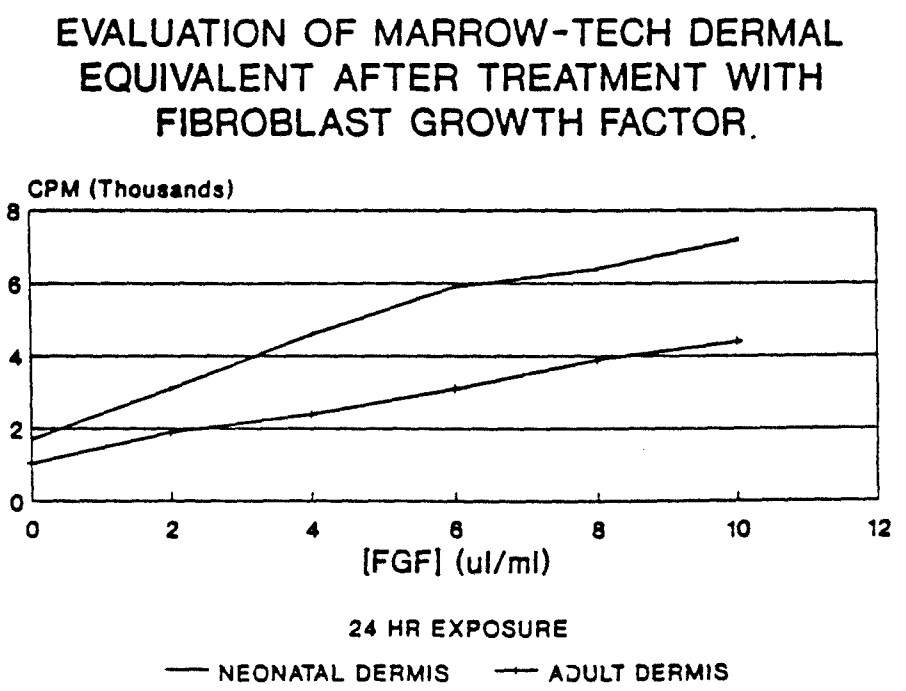
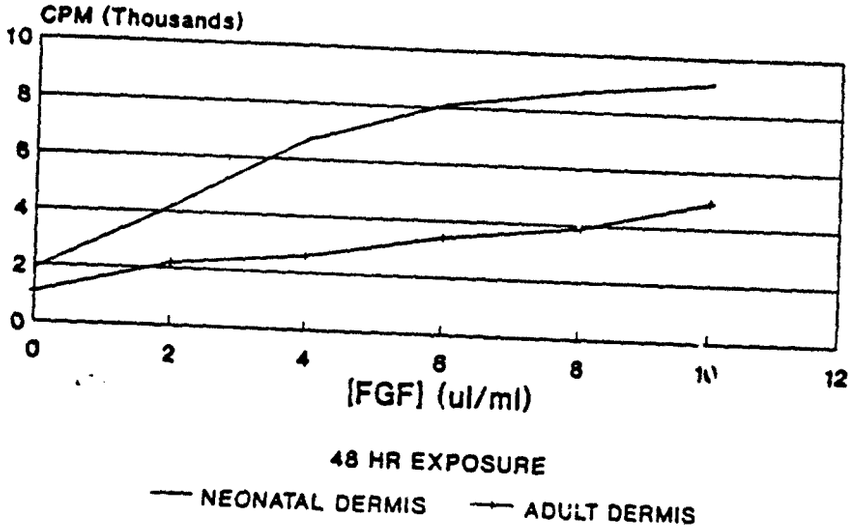


FIGURE 2



FGFDE24

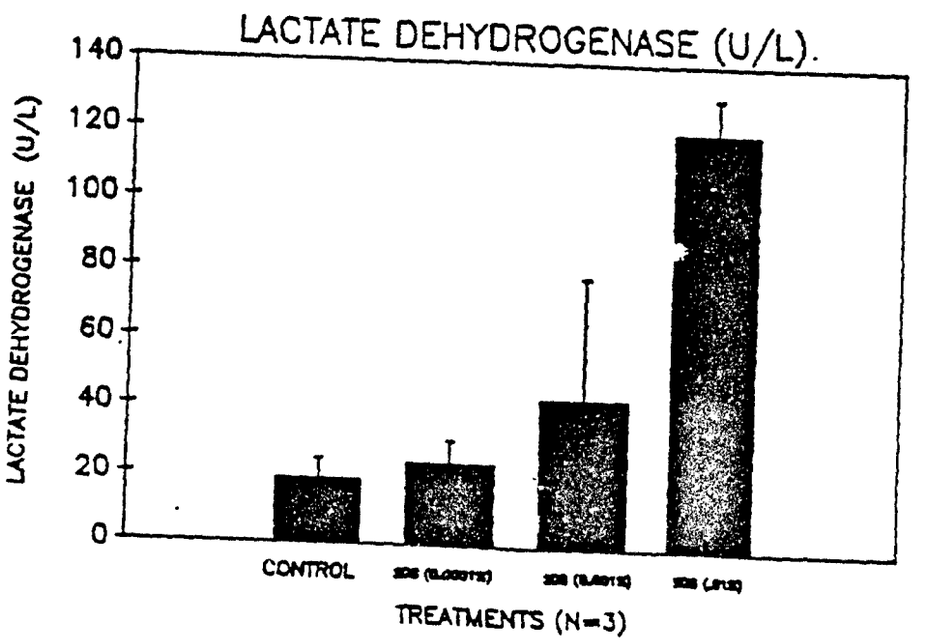
EVALUATION OF MARROW-TECH DERMAL EQUIVALENT AFTER TREATMENT WITH FIBROBLAST GROWTH FACTOR



FGFDE48

FIGURE 4

EVALUATION OF MARROW -TECH FIBROBLAST/KERATINOCYTE CULTURES AFTER SDS TREATMENT



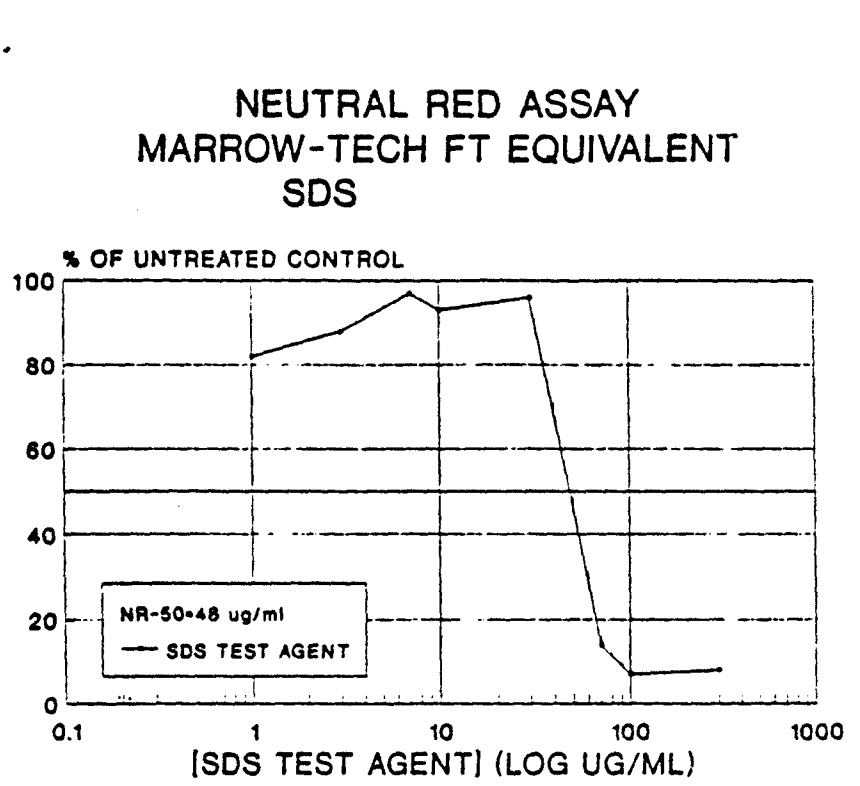

NEUTRAL RED ASSAY
MARROW-TECH FT EQUIVALENT
SDS
% OF UNTREATED CONTROL
100
80
60
40
20
0
NR-50-48 ug/ml
SDS TEST AGENT
0.1
1
10
100
1000
[SDS TEST AGENT] (LOG UG/ML)
102689S

FIGURE 6

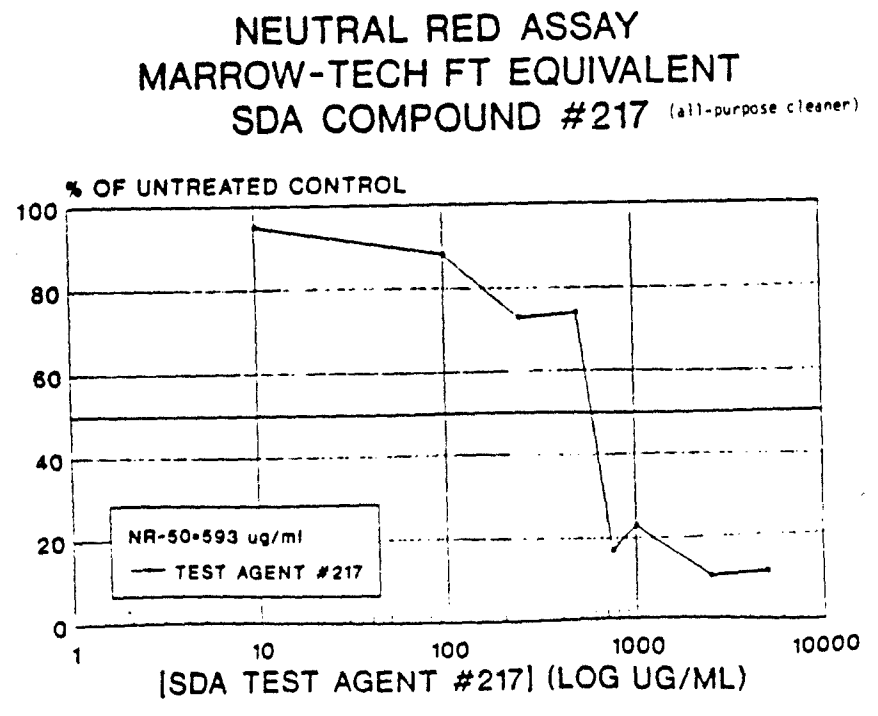
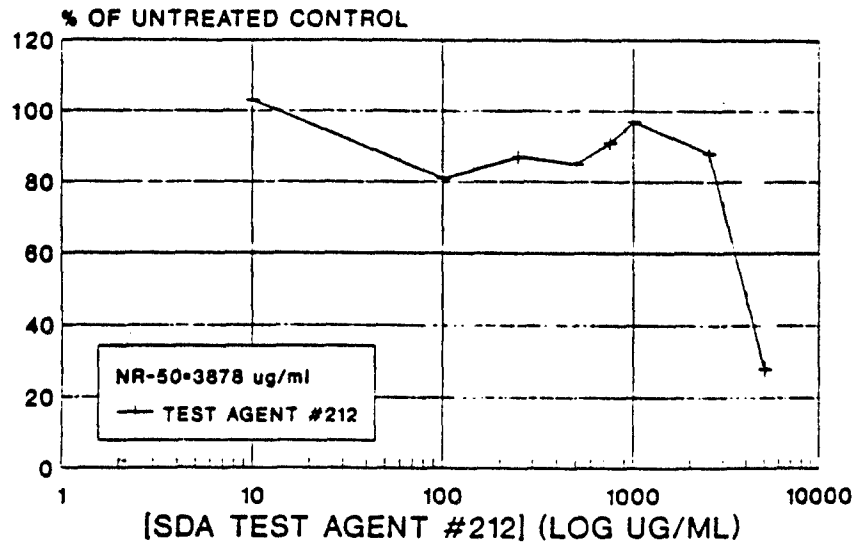

NEUTRAL RED ASSAY
MARROW-TECH FT EQUIVALENT
SDA COMPOUND #217 (all-purpose cleaner)
% OF UNTREATED CONTROL
100
80
60
40
20
0
NR-50-593 ug/ml
TEST AGENT #217
1
10
100
1000
10000
[SDA TEST AGENT #217] (LOG UG/ML)
102689B

FIGURE 7

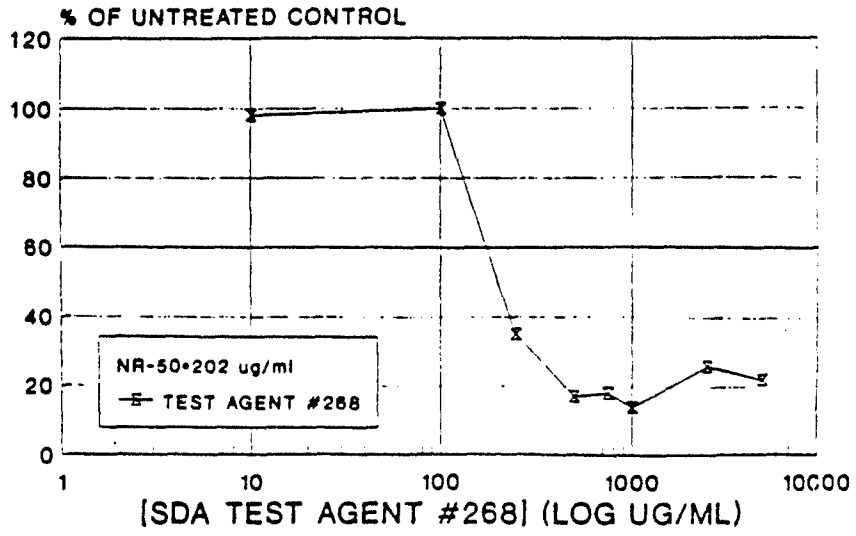
NEUTRAL RED ASSAY
MARROW-TECH FT EQUIVALENT
SDA COMPOUND #212 (light duty liquid cleaner)



102689C

FIGURE 8

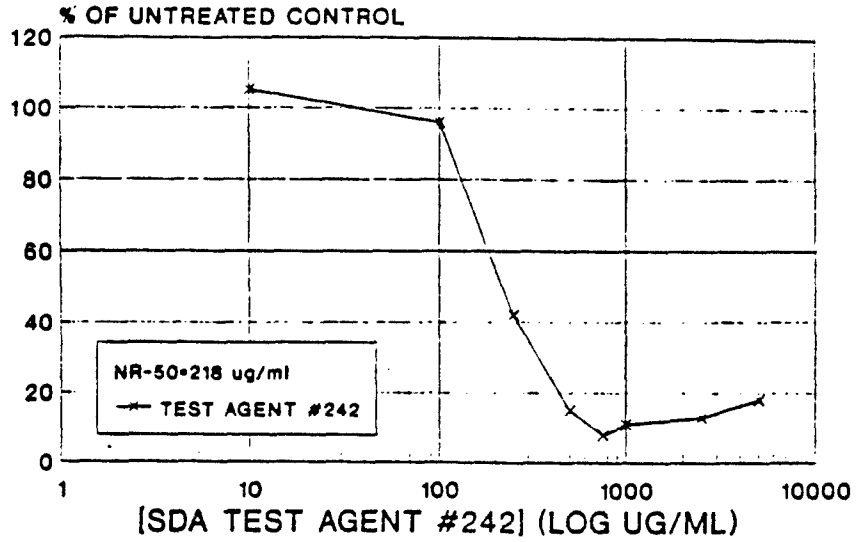
NEUTRAL RED ASSAY
MARROW-TECH FT EQUIVALENT
SDA COMPOUND #268 (Sodium Lauryl Sulfate 29%)



1026268

FIGURE 9

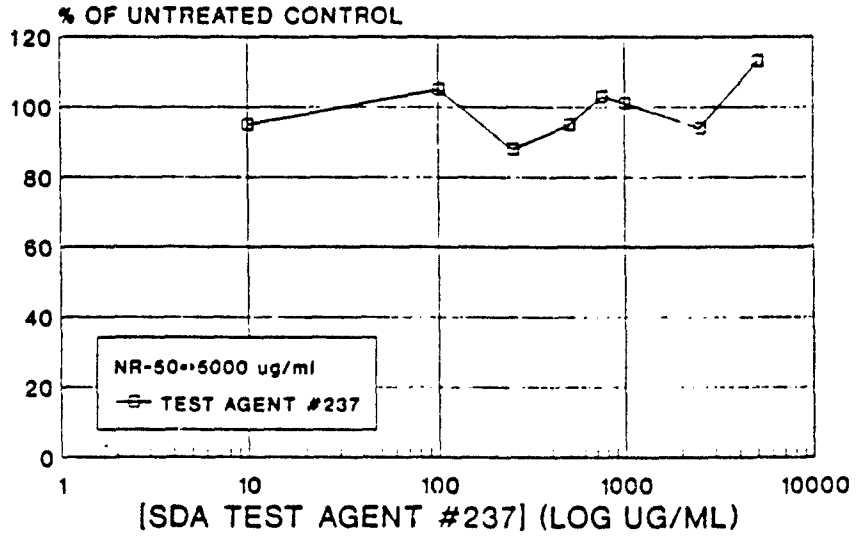
NEUTRAL RED ASSAY
MARROW-TECH FT EQUIVALENT
SDA COMPOUND #242 (light duty liquid detergent)



1026242

FIGURE 10

NEUTRAL RED ASSAY
MARROW-TECH FT EQUIVALENT
SDA COMPOUND #237 (Acetic Acid 3%)

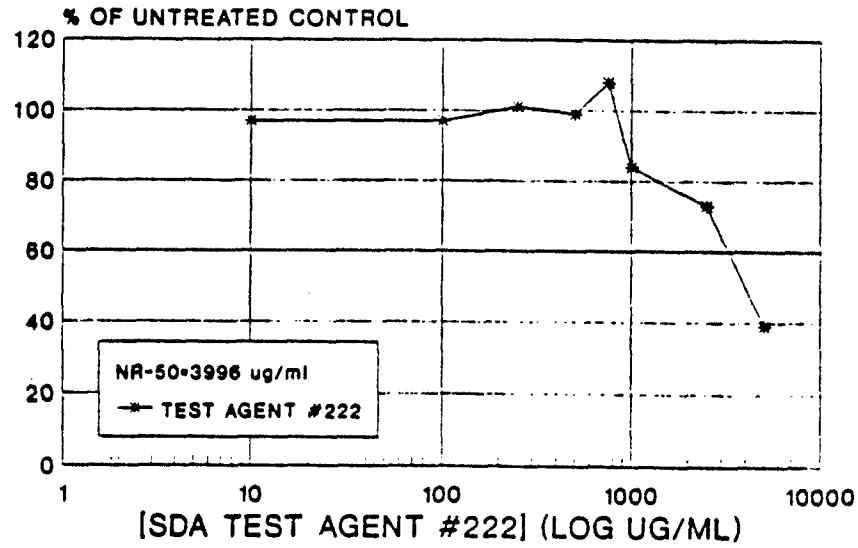


1026237

FIGURE 11

NEUTRAL RED ASSAY
MARROW-TECH FT EQUIVALENT
SDA COMPOUND #222

(Ammonia surfactant solution)

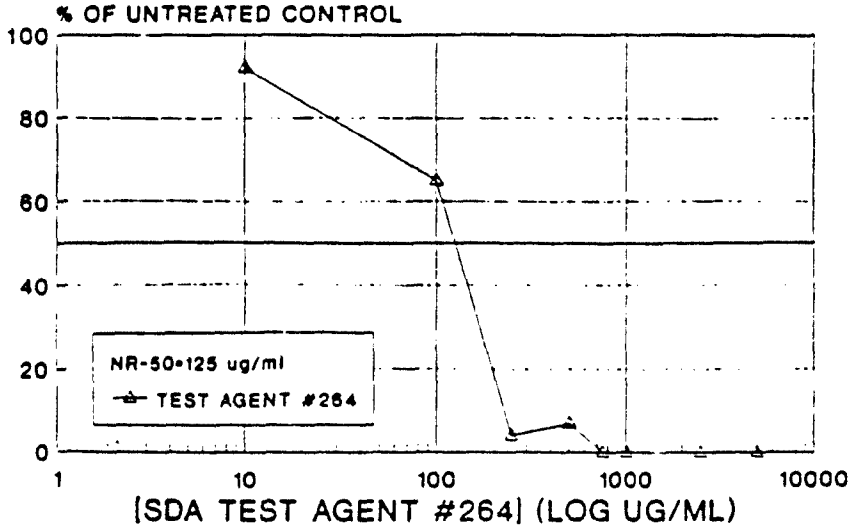


102689D

FIGURE 12

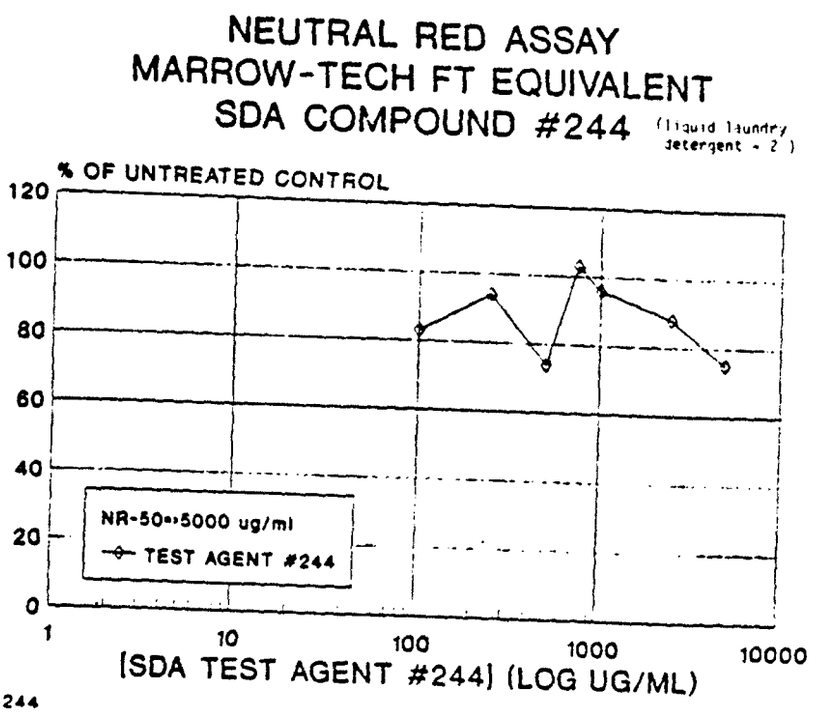
NEUTRAL RED ASSAY
MARROW-TECH FT EQUIVALENT
SDA COMPOUND #264

(liquid laundry detergent with fabric softener)



1026264

FIGURE 13



1026244

FIGURE 14

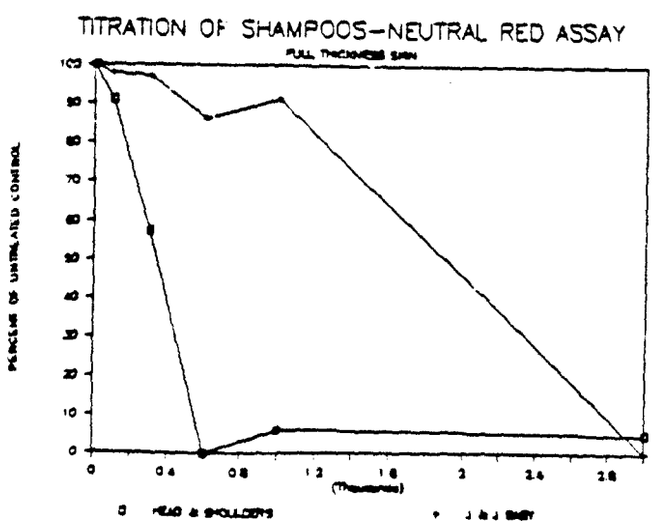


FIGURE 15

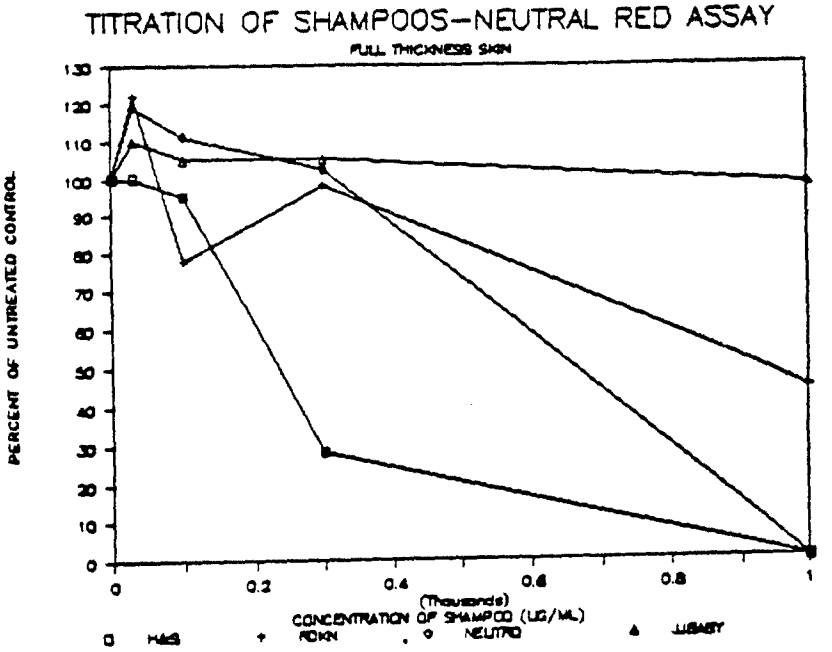


FIGURE 15

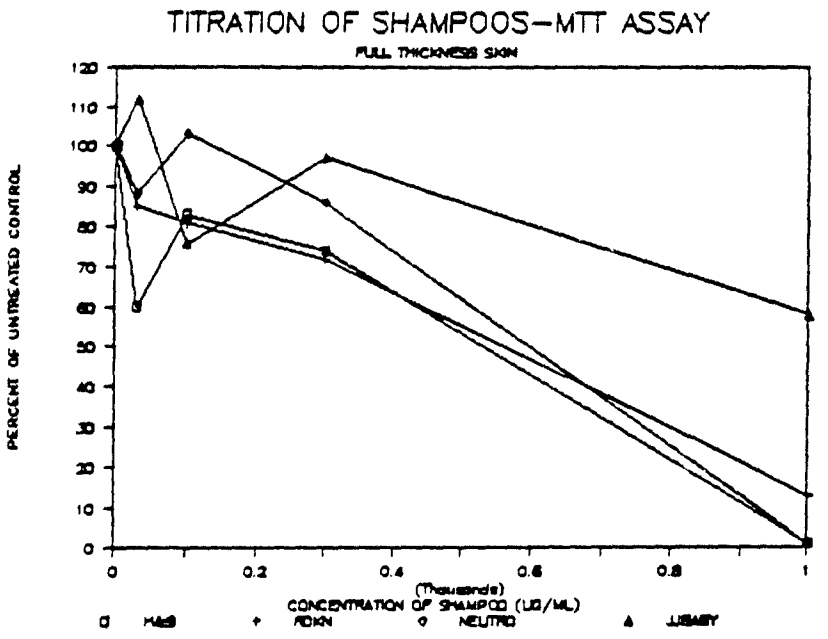


FIGURE 17

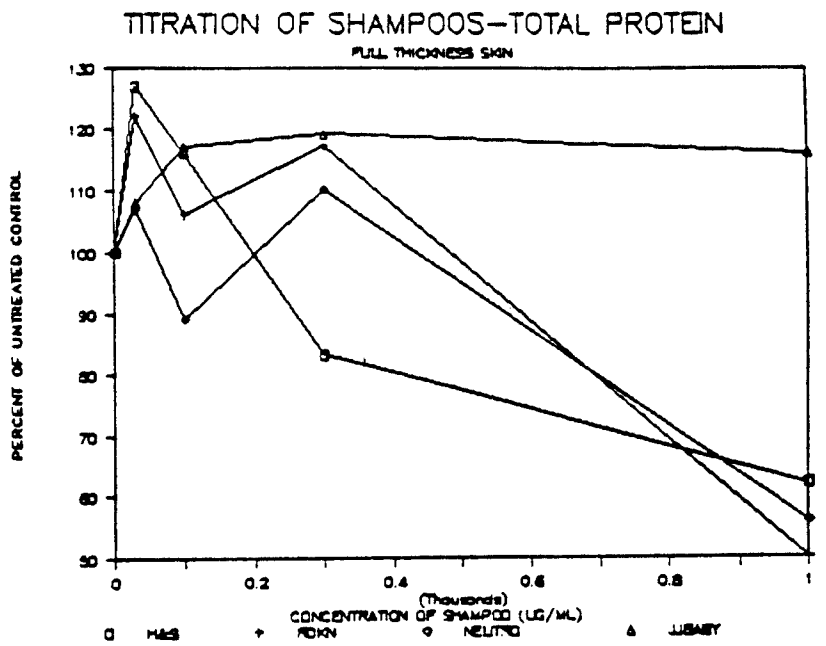


FIGURE 18

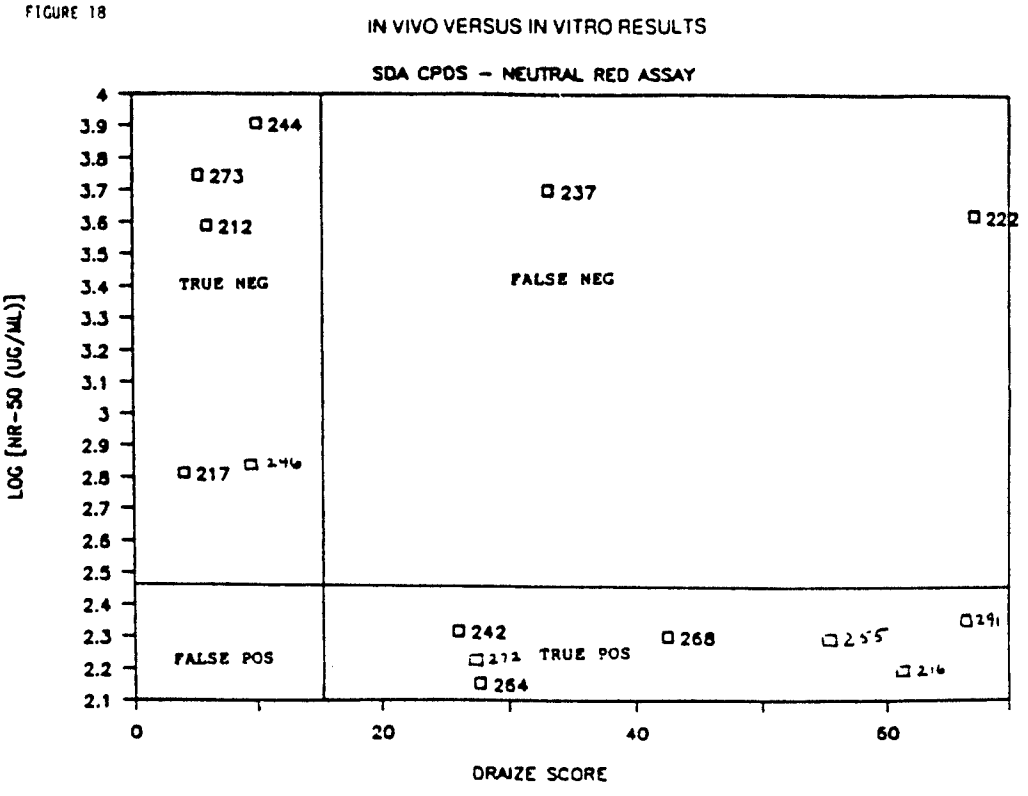


FIGURE 19

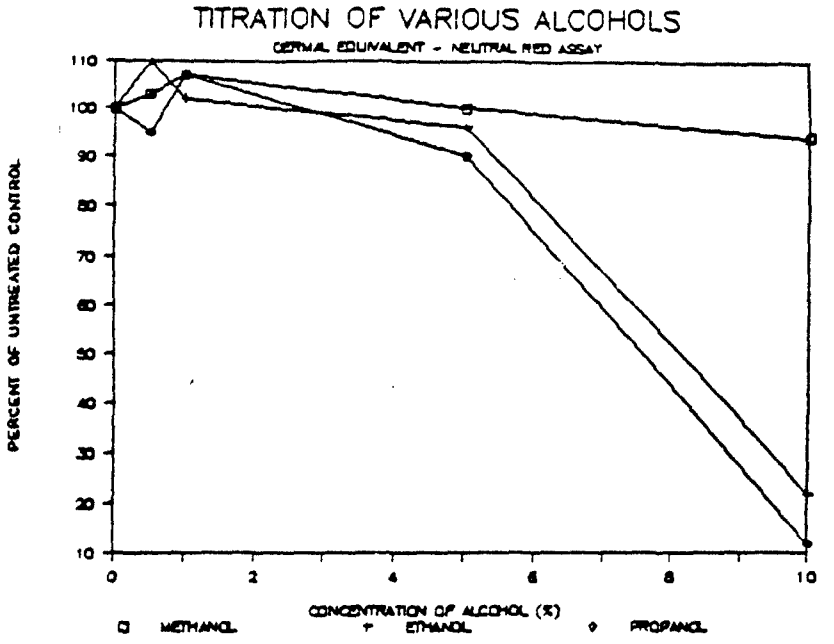


FIGURE 20

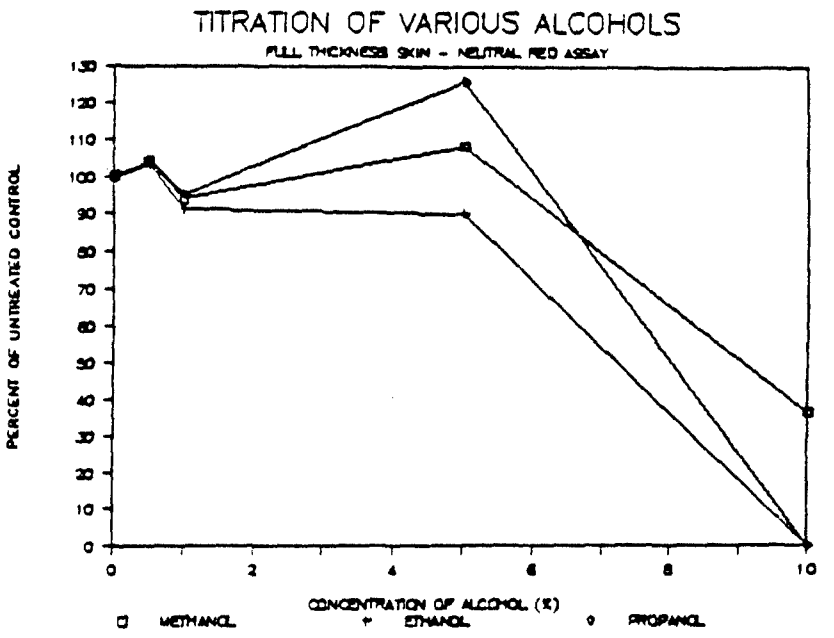


FIGURE 21

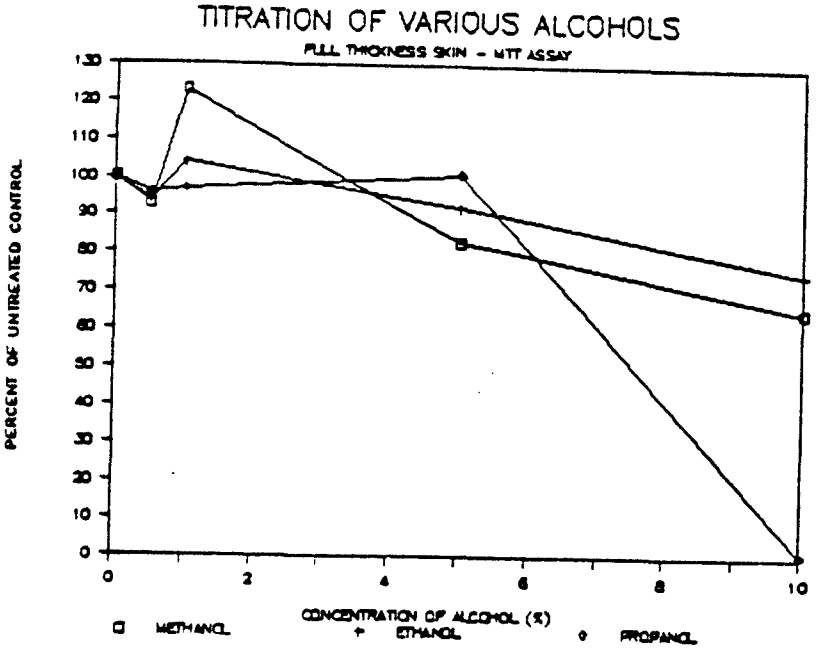


FIGURE 22

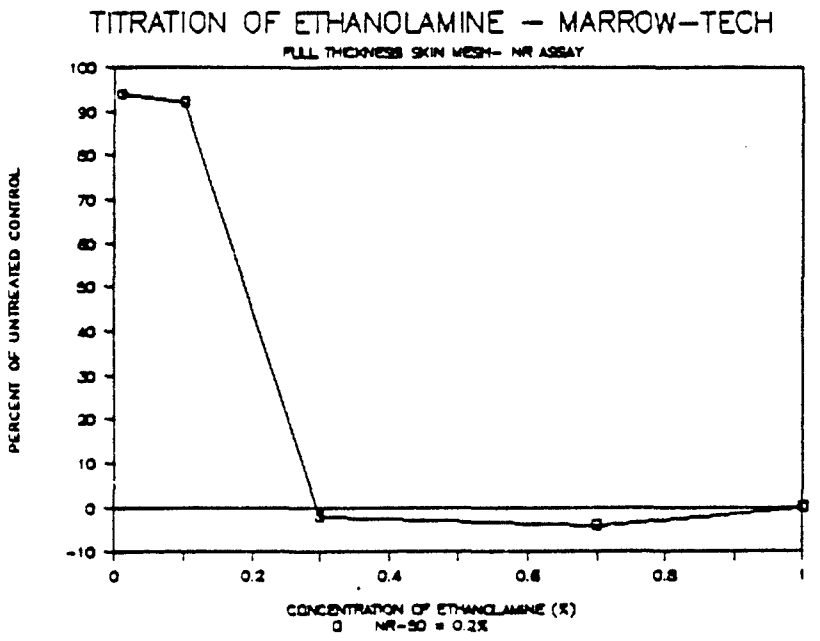
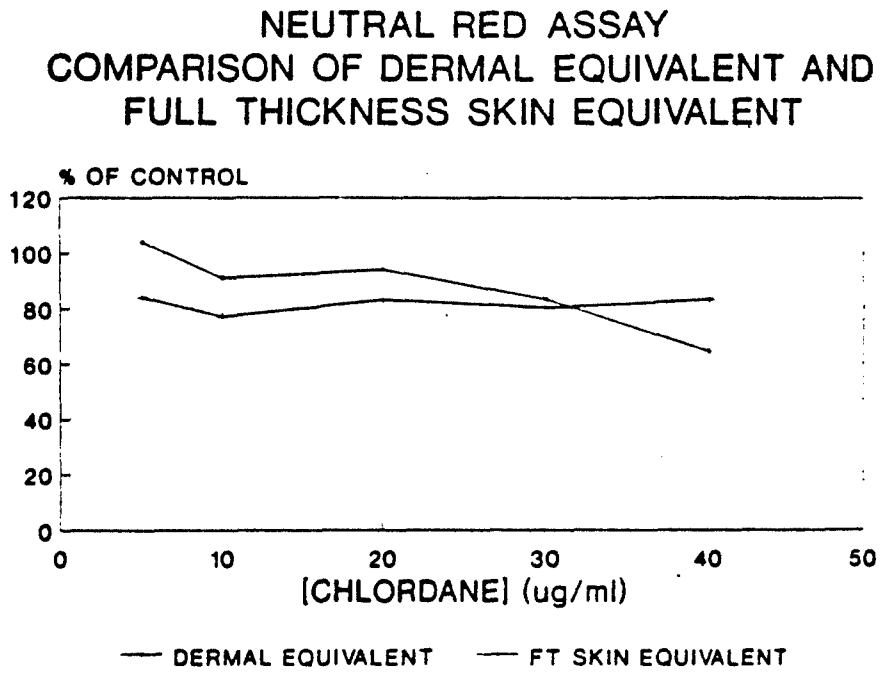
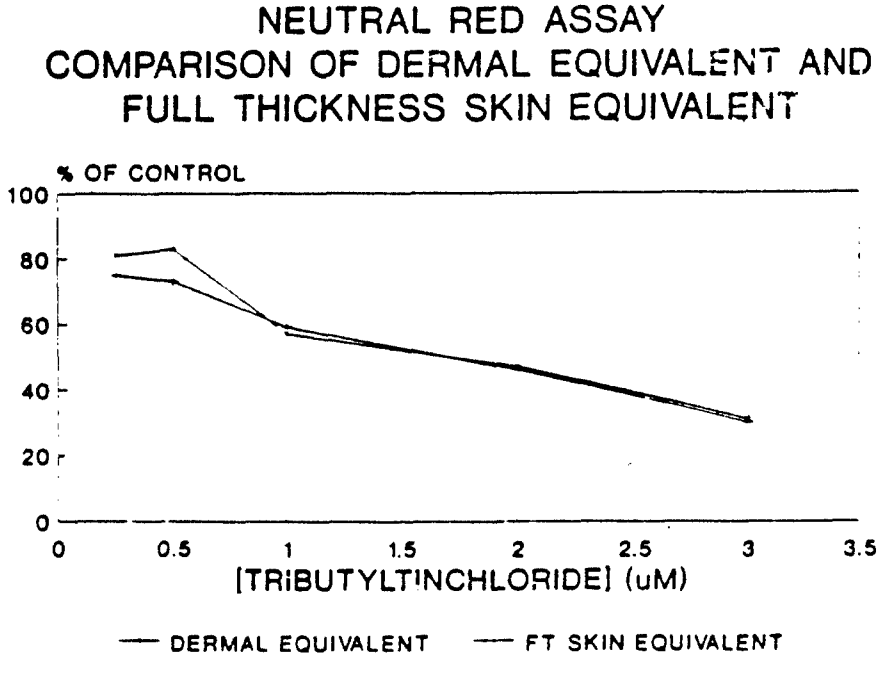


FIGURE 23



MTICHLOR

FIGURE 24



MTITRICH

FIGURE 25

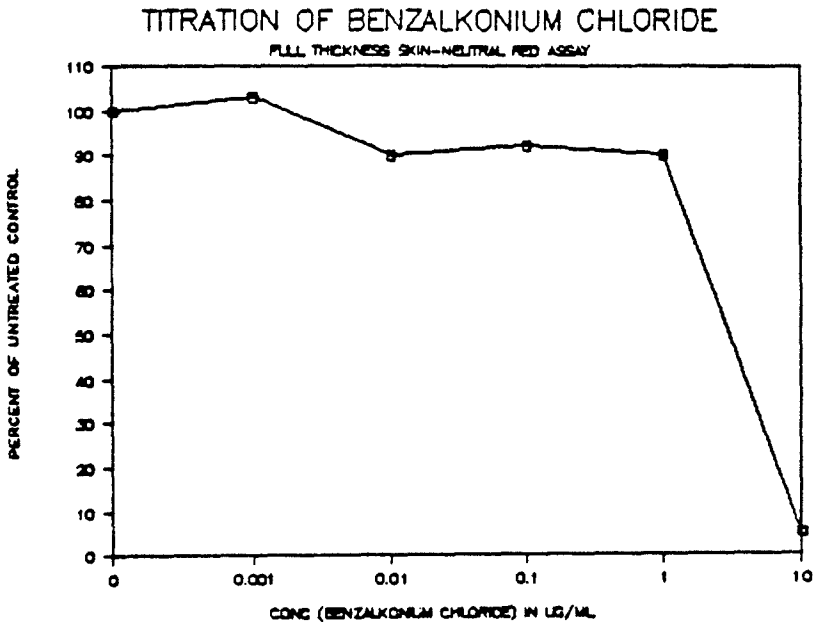


FIGURE 26

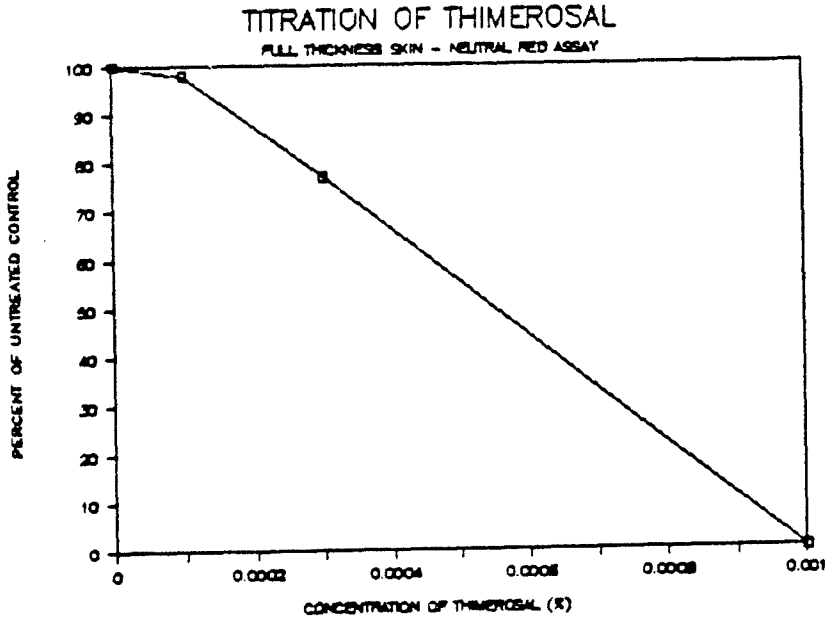
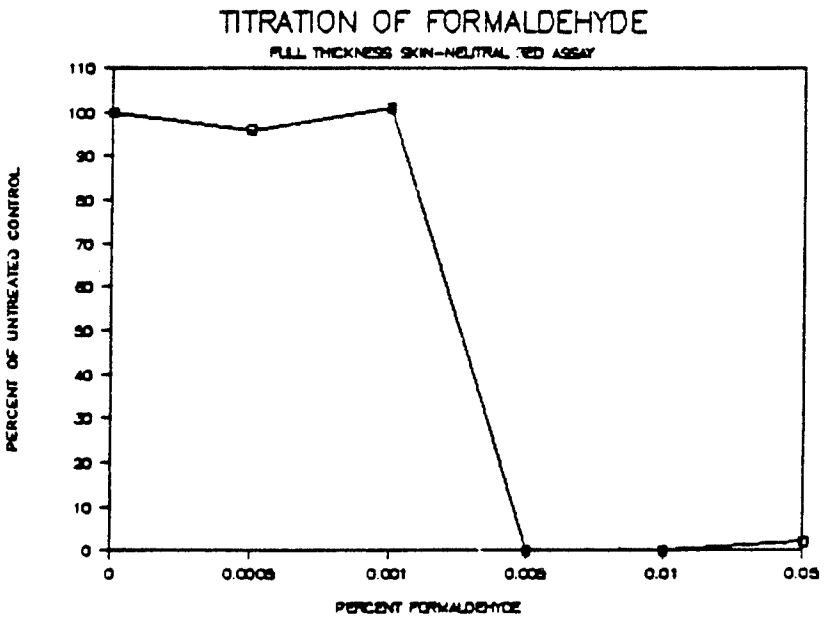


FIGURE 27


TITRATION OF FORMALDEHYDE
FULL THICKNESS SKIN-NEUTRAL RED ASSAY
PERCENT OF UNTREATED CONTROL
110
100
90
80
70
60
50
40
30
20
10
0
0
0.0005
0.001
0.005
0.01
0.05
PERCENT FORMALDEHYDE